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PATENT- OG VAREMÆRKESTYRELSEN

New insertion sites

The present invention relates to recombinant Modified Vaccinia Ankara (MVA) viruses and, particularly, to novel insertion sites useful for the integration of exogenous sequences into the MVA genome

Background of the invention

Modified Vaccinia Ankara (MVA) is a member of the Ortopoxvirus family and has been generated by about 570 serial passages on chicken embryo fibroblasts of the Ankara strain of Vaccinia virus (CVA) (for review see Mayr, A , *et al* [1975], Infection 3, 6-14) As a consequence of these passages the resulting MVA virus contains 31 kilobases less genomic information compared to CVA and is highly host cell restricted (Meyer, H *et al* , J Gen Virol 72, 1031-1038 [1991]) MVA is characterized by its extreme attenuation, namely a diminished virulence or infectiosity but still an excellent immunogenicity When tested in a variety of animal models, MVA was proven to be avirulent even in immuno-suppressed individuals More importantly, the excellent properties of the MVA strain have been demonstrated in extensive clinical trials (Mayr *et al* , Zbl Bakt Hyg I, Abt Org B 167, 375-390 [1987]) During these studies in over 120,000 humans, including high risk patients, no side effects were seen (Stickl *et al* , Dtsch med Wschr 99, 2386-2392 [1974])

It has been further found that MVA is blocked in the late stage of the virus replication cycle in mammalian cells (Sutter, G and Moss, B [1992] Proc Natl Acad Sci

USA 89, 10847-10851) Accordingly, MVA fully replicates its DNA, synthesizes early, intermediate and late gene products, but is not capable to assemble mature infectious virions, which could be released from an infected cell For this reason, namely to be replication restricted, MVA was proposed to serve as a gene expression vector

More recently, MVA was used to generate recombinant vaccines, expressing antigenic sequences inserted either at the site of the tymidine-kinase (tk) gene (US 5,185,146) or at the site of a naturally occurring deletion within the MVA genome (PCT/EP96/02926)

Although the tk insertion locus is widely used for the generation of recombinant poxviruses, particularly for the generation of recombinant Vaccinia viruses (Mackett, *et al* [1982] P N A S USA 79, 7415-7419) this technology was not applicable for MVA It was shown by Scheiflinger et al , that MVA is much more sensible to modifications of the genome compared to other poxviruses, which can be used for the generation of recombinant poxviruses Scheiflinger et al showed in particular that one of the most commonly used site for the integration of heterologous DNA into poxviral genomes, namely the thymdine kinase (tk) gene locus, cannot be used to generate recombinant MVA Any resulting tk(-) recombinant MVA proofed to be highly unstable and upon purification immediately deleted the inserted DNA together with parts of the genomic DNA of MVA (Scheiflinger et al [1996], Arch Virol 141 pp 663-669)

Instability and, thus, high probability of genomic recombination is a known problem within pox virology Actually, MVA was established during long-term passages

exploiting the fact that the viral genome of CVA is instable Several thousands of nucleotides (31 kb) had been deleted from the MVA genome, which therefore is characterized by 6 major and numerous small deletions in comparison to the original CVA genome

The genomic organization of the MVA genome has been described recently (Antoine *et al* [1998], Virology 244, 365-396) The 178 kb genome of MVA is densely packed and comprises 193 individual open reading frames (ORF), which code for proteins of at least 63 amino acids in length In comparison with the highly infectious Variola virus and also the prototype of Vaccinia virus, namely the strain Copenhagen, the majority of ORF's of MVA are fragmented or truncated (Antoine *et al* [1998], Virology 244, 365-396) However, with very few exceptions all ORF's, including the fragmented and truncated ORF's, get transcribed and translated into proteins In the following to describe the invention the nomenclature of Antoine et al is used and - where appropriated - the nomenclature based on Hind III restriction enzyme digest indicated in brackets

So far, only the insertion of exogenous DNA into the naturally occurring deletion sites of the MVA genome led to stable recombinant MVA's (PCT/EP96/02926) Unfortunately, there is only a restricted number of naturally occurring deletion sites in the MVA genome Additionally it was shown that other insertion sites, such as e g the tk gene locus, are hardly useful for the generation of recombinant MVA (Scheiflinger et al [1996], Arch Virol 141 pp 663-669)

Object of the invention

Therefore, it is an object of the present invention to provide new insertion vectors, which will direct the insertion of exogenous sequences into newly identified insertion sites of the MVA genome

It is a further object of the present invention to provide recombinant MVA, which comprises exogenous DNA sequences stably integrated into new insertion sites of the MVA genome

Detailed description of the invention

The inventors of the present invention identified new sites for the insertion of exogenous DNA into the genome of Modified Vaccinia Ankara (MVA) The new insertion sites are located in the intergenic region (IR) between adjacent open reading frames (ORF) of the MVA genome While the ORFs encode for proteins whether essential or unessential for the viral life circle, the IRs between two ORFs have no coding capacity, but may comprise transcription control elements, such as promoter and enhancer sequences, or binding sites involved in the transcriptional control of the viral gene expression Thus, the IR may be involved in the regulatory control of the viral life cycle Accordingly, the present invention provides recombinant MVA, which comprise one or more exogenous DNA sequences inserted into an IR between two adjacent ORF The inventors have shown that the new insertion sites have, thus, the unexpected advantage that exogenous DNA sequences can be stably inserted into the MVA genome without influencing or changing the typical characteristics and gene expression of MVA The new

insertion sites are especially useful, since no ORF or coding sequence of MVA is altered

Depending on the orientation of the two adjacent ORFs the IR - in between these ORFs - is flanked either by the two stop codons of the two adjacent ORFs, by the two start codons of the two adjacent ORFs, by the stop codon of the first ORF and the start codon of the second ORF or by the start codon of the first ORF and the stop codon of the second ORF

According to a preferred embodiment of the present invention the insertion site for the exogenous DNA into the IR is downstream or 3' of the stop codon of a first ORF In case the adjacent ORF, also termed second ORF, has the same orientation as the first ORF, this insertion site downstream of the stop codon of the first ORF contemporarily lies upstream or 5' of the start codon of the second ORF In case the orientation of the first and the second ORF is not from the left to the right (5' -> 3'), but is from the right to the left (3' <- 5'), the insertion site is located upstream of the start codon of the first ORF and downstream of the stop codon of the second ORF

In case the second ORF has an opposite orientation relative to the first ORF, which means the orientation of the two adjacent ORFs points to each other, then the insertion site lies downstream of the stop codons of both ORFs

As a third alternative, in case the two adjacent ORF read in opposite direction, but the orientation of the two adjacent ORFs points away from each other, which is synonymous with a positioning that is characterized in that the start codons of the two adjacent ORF point to

each other, then the exogenous DNA is inserted upstream relative to both start codons

According to further specific embodiments of the present invention the exogenous DNA is inserted downstream of one of the following ORFs 005R, 006L (corresponding to C10L), 007R, 008L, 019L (corresponding to C6L), 020L (corresponding to N1L), 021L (corresponding to N2L), 023L (corresponding to K2L), 028R (corresponding to K7R), 029L (corresponding to F1L), 037L (corresponding to F8L), 045L (corresponding to F15L), 050L (corresponding to E3L), 052R (corresponding to E5R), 054R (corresponding to E7R), 055R (corresponding to E8R), 056L (corresponding to E9L), 057R (corresponding to E10R), 058L (corresponding to E11L), 062L (corresponding to I1L), 065L (corresponding to I4L), 069R (corresponding to I8R), 070L (corresponding to G1L), 081R (corresponding to L2R), 082L (corresponding to L3L), 086R (corresponding to J2R), 088R (corresponding to J4R), 089L (corresponding to J5L), 092R (corresponding to H2R), 093L (corresponding to H3L), 107R (corresponding to D10R), 108L (corresponding to D11L), 122R (corresponding to A11R), 123L (corresponding to A12L), 125L (corresponding to A14L), 126L (corresponding to A15L), 135R (corresponding to A24R), 136L (corresponding to A25L), 137L (corresponding to A26L), 141L (corresponding to A30L), 148R (corresponding to A37R), 149L (corresponding to A38L), 152R (corresponding to A40R), 153L (corresponding to A41L), 156R, 157L (corresponding to A44L), 159R (corresponding to A46R), 160L (corresponding to A47L), 165R (corresponding to A56R), 166R (corresponding to A57R), 167R (corresponding to B1R), 169R (corresponding to B2R), 170R (corresponding to B3R), 176R (corresponding to B8R), 180R (corresponding to B12R), 184R (corresponding to B16R), 185L

(corresponding to B17L), 187R (corresponding to B19R), 188R or 191R (corresponding to B23R)

The exogenous DNA is inserted in the IR upstream of the start codon of one of the following ORFs 007R, 028R, 090R, 095R (corresponding to H5R), 154R (corresponding to A42R)

According to a further embodiment of the present invention the exogenous DNA sequence comprises at least one coding sequence The coding sequence in the exogenous DNA is operably linked to a transcription control element, preferably a poxviral transcription control element Additionally, also combinations between poxviral transcription control element and e g internal ribosomal entry sites can be used

According to a further embodiment the exogenous DNA sequence can also comprise two or more coding sequences linked to one or several transcription control elements Preferably the coding sequence encodes one or more proteins, polypeptides, peptides, foreign antigens or antigenic epitopes of therapeutically interesting genes

Therapeutically interesting genes according to the present invention are genes derived from or homologous to genes of pathogenous or infectious microorganism, which are disease causing Accordingly, in the context of the present invention such therapeutically interesting genes are presented to the immune system of an organism in order to affect, preferably induce a specific immune response and, thereby, vaccinate or prophylactically protect the organism against an infection with the microorganism In further preferred embodiments of the present invention the therapeutically interesting genes are selected from genes of infectious viruses, e g - but

not limited to - Dengue virus, Japanese encephalitis virus, Hepatitis virus B or C, or immunodeficiency viruses such as HIV

Furthermore, therapeutically interesting genes according to the present invention also comprise disease related genes, which have a therapeutic effect on proliferative disorder, cancer or metabolic diseases For example, a therapeutically interesting gene regarding cancer could be a cancer antigen that has the capacity to induce a specific anti-cancer immune reaction

According to a further embodiment of the present invention the coding sequence comprises at least one marker or selection gene

Selection genes transduce a particular resistance to a cell whereby a certain selection method becomes possible The skilled practitioner is familiar with a variety of selection genes, which can be used in a poxviral system Among these are e g Neomycin resistance gene (NPT) or Phosphribosyl transferase gene (gpt)

Marker genes induce a colour reaction in transduced cells, which can be used to identify transduced cells The skilled practitioner is familiar with a variety of marker genes, which can be used in a poxviral system Among these are the gene encoding e g ß-Galactosidase (ß-gal), ß-Glucosidase (ß-glu) or Green Fluorescent protein (EGFP)

According to still a further embodiment of the present invention the exogenous DNA sequence comprises a spacing sequence, which separates poxviral transcription control element and/or coding sequence in the exogenous DNA sequence from the stop codon and/or the start codon of the adjacent ORFs This spacer sequence between the

stop/start codon of the adjacent ORF and the inserted coding sequence in the exogenous DNA has the advantage to stabilize the inserted exogenous DNA and, thus, any resulting recombinant virus The size of the spacer sequence is variable as long as the sequence is without own coding or regulatory function

According to a further embodiment the spacer sequence separating the poxviral transcription control element and/or the coding sequence in the exogenous DNA sequence from the stop codon of the adjacent ORF is at least one nucleotide long

According to another embodiment of the present invention the spacing sequence separating the poxviral transcription control element and/or the coding sequence in the exogenous DNA sequence from the start codon of the adjacent ORF is at least 30 nucleotides Particularly, in cases were a typical Vaccinia virus promoter element is identified upstream of a start codon the insertion of exogenous DNA may not separate the promoter element from the start codon of the adjacent ORF A typical Vaccinia promoter element can be identified by scanning for e g the sequence "TAAAT for late promoters (Davison & Moss, J Mol Biol 1989, 210 771-784) and an A/T rich domain for early promoters A spacing sequence of about 30 nucleotides is the preferred distance to secure that a poxviral promoter located upstream of the start codon of the ORF is not influenced Additionally, according to a further preferred embodiment the distance between the inserted exogenous DNA and the start codon of the adjacent ORF is around 50 nucleotides and more preferably around 100 nucleotides

According to a further preferred embodiment of the present invention, the spacing sequence comprises an additional poxviral transcription control element, which is capable to control the transcription of the adjacent ORF

The recombinant MVA according to the present invention is useful as a medicament or vaccine It is according to a further embodiment used for the introduction of the exogenous coding sequence into a target cell, said sequence being either homologous or heterologous to the target cell

The introduction of an exogenous coding sequence into a target cell may be done *in vitro* to produce proteins, polypeptides, peptides or antigenic epitopes This method comprises the infection of a host cell with the recombinant MVA according to the invention, cultivation of the infected host cell under suitable conditions, and isolation and/or enrichment of the peptide, protein and/or virus produced by said host cell

Furthermore, the method for introduction of one or more homologous or one or more heterologous sequence into cells may be applied for *in vitro* and *in vivo* therapy For *in vitro* therapy, isolated cells that have been previously *(ex vivo)* infected with the recombinant MVA according to the invention are administered to the living animal body for affecting, preferably inducing an immune response For *in vivo* therapy, the recombinant poxvirus according to the invention is directly administered to the living animal body for affecting, preferably inducing an immune response In this case, the cells surrounding the site of inoculation, but also cells where the virus is transported to via e g the blood stream, are directly

infected *in vivo* by the recombinant MVA according to the invention After infection these cells synthesize the proteins, peptides or antigenic epitopes of the therapeutic genes, which are encoded by the exogenous coding sequences and, subsequently, present them or parts thereof on the cellular surface Specialized cells of the immune system recognize the presentation of such heterologous proteins, peptides or epitopes and launch a specific immune response

Since the MVA is highly growth restricted and, thus, highly attenuated, it is useful for the treatment of a wide range of mammals including humans, including immune-compromised humans The present invention also provides pharmaceutical compositions and vaccines for inducing an immune response in a living animal body, including a human

The pharmaceutical composition may generally include one or more pharmaceutical acceptable and/or approved carriers, additives, antibiotics, preservatives, adjuvants, diluents and/or stabilizers Such auxiliary substances can be water, saline, glycerol, ethanol, wetting or emulsifying agents, pH buffering substances, or the like Suitable carriers are typically large, slowly metabolized molecules such as proteins, polysaccharides, polylactic acids, polyglycollic acids, polymeric amino acids, amino acid copolymers, lipid aggregates, or the like

For the preparation of vaccines, the recombinant poxvirus according to the invention is converted into a physiologically acceptable form This can be done based on the experience in the preparation of poxvirus vaccines used for vaccination against smallpox (as described by

Stickl, H *et al* [1974] Dtsch med Wschr 99, 2386-2392) For example, the purified virus is stored at -80°C with a titre of 5x10E8 $TCID_{50}$/ml formulated in about 10mM Tris, 140 mM NaCl pH 7 4 For the preparation of vaccine shots, e g , 10E2-10E8 particles of the virus are lyophilized in 100 ml of phosphate-buffered saline (PBS) in the presence of 2% peptone and 1% human albumin in an ampoule, preferably a glass ampoule Alternatively, the vaccine shots can be produced by stepwise freeze-drying of the virus in a formulation This formulation can contain additional additives such as mannitol, dextran, sugar, glycine, lactose or polyvinylpyrrolidone or other aids such as antioxidants or inert gas, stabilizers or recombinant proteins (e g human serum albumin) suitable for *in vivo* administration The glass ampoule is then sealed and can be stored between 4°C and room temperature for several months However, as long as no need exists the ampoule is stored preferably at temperatures below -20°C

For vaccination or therapy the lyophilisate can be dissolved in 0 1 to 0 5 ml of an aqueous solution, preferably physiological saline or Tris buffer, and administered either systemically or locally, i e parenterally, subcutaneous, intramuscularly, by scarification or any other path of administration know to the skilled practitioner The mode of administration, the dose and the number of administrations can be optimized by those skilled in the art in a known manner However, most commonly a patient is vaccinated with a second shot about one month to six weeks after the first vaccination shot

The present invention further comprises the plasmid vectors, which can be used to generate recombinant MVA according to the present invention

The plasmid vector according to the present invention comprise a DNA sequence derived from the genome of MVA, wherein said DNA sequence comprises a complete or partial fragment of an IR between two adjacent ORF of the viral genome This complete or partial fragment of an IR is used in the plasmid vector to direct the insertion of exogenous DNA sequences to particular sites in the MVA genome, namely the corresponding IR Preferably, the plasmid vector comprises inserted into said IR-derived sequence at least one cloning site for the insertion of a poxviral transcription control element and a exogenous DNA sequence of interest Optionally, the plasmid vector comprises a reporter- and/or selection gene cassette

According to further preferred embodiments the complete or partial fragment of the IR, which is used in the plasmid vector to direct the insertion of exogenous DNA sequences into the MVA genome, is adjacent to one of the following ORFs 005R, 006L, 007R, 008L, 019L, 020L, 021L, 023L, 028R, 029L, 037L, 045L, 050L, 052R, 054R, 055R, 056L, 057R, 058L, 062L, 065L, 069R, 070L, 081R, 082L, 086R, 088R, 089L, 090R, 092R, 093L, 095R, 107R, 108L, 122R, 123L, 125L, 126L, 135R, 136L, 137L, 141L, 148R, 149L, 152R, 153L, 154R, 156R, 157L, 159R, 160L, 165R, 166R, 167R, 169R, 170R, 176R, 180R, 184R, 185L, 187R, 188R and 191R Optionally, the plasmid vector according to the present invention also comprises one or more ORFs selected from the above-mentioned selection

To generate a plasmid vector according to the present invention the complete or partial IR sequences adjacent

to the insertion site are isolated and cloned into a standard cloning vector, such as pBluescript (Stratagene), wherein they flank the exogenous DNA to be inserted into the MVA genome Optionally, such an plasmid vector comprises a selection- or reporter gene cassette, which due to a repetitive sequence can be deleted from the final recombinant virus

Methods to introduce exogenous DNA sequences by an plasmid vector into a MVA genome and methods to obtain recombinant MVA are well known to the person skilled in the art and, additionally, can be deduced from the following references

- *Molecular Cloning*, A laboratory Manual Second Edition By J Sambrook, E F Fritsch and T Maniatis Cold Spring Harbor Laboratory Press 1989 Describes techniques and know how for standard molecular biology techniques such cloning of DNA, RNA isolation, western blot analysis, RT-PCR and PCR amplification techniques,
- *Virology Methods Manual* Edited by Brian WJ Mahy and Hillar O Kangro Academic Press 1996 Describes techniques for the handling and manipulation of viruses,
- *Molecular Virology* A Practical Approach Edited by AJ Davison and RM Elliott The Practical Approach Series IRL Press at Oxford University Press Oxford 1993 Chapter 9 Expression of genes by Vaccinia virus vectors,
- *Current Protocols in Molecular Biology* Publisher John Wiley and Son Inc 1998 Chapter 16, section IV Expression of proteins in mammalian cells using Vaccinia viral vector Describes techniques and know-how for the handling, manipulation and genetic engineering of MVA

According to still another embodiment the invention includes the DNA sequence or parts thereof derived from

or homologous to the MVA according to the invention This DNA sequence consists of fragments of the genome of the MVA according to the invention comprising a complete or partial fragment of an IR between two adjacent ORF of the viral genome and comprising a DNA sequence, preferably parts of the exogenous DNA sequence, inserted into said IR

According to further embodiments the DNA sequence comprise complete or partial fragments of the IR adjacent to one of the following ORFs 005R, 006L, 007R, 008L, 019L, 020L, 021L, 023L, 028R, 029L, 037L, 045L, 050L, 052R, 054R, 055R, 056L, 057R, 058L, 062L, 065L, 069R, 070L, 081R, 082L, 086R, 088R, 089L, 090R, 092R, 093L, 095R, 107R, 108L, 122R, 123L, 125L, 126L, 135R, 136L, 137L, 141L, 148R, 149L, 152R, 153L, 154R, 156R, 157L, 159R, 160L, 165R, 166R, 167R, 169R, 170R, 176R, 180R, 184R, 185L, 187R, 188R and 191R

The DNA sequences according to the invention can be used to identify or isolate the MVA or its derivatives according to the invention, cells or individuals infected with a MVA according to the present invention The DNA sequences are e g used to generate PCR-primers, hybridization probes or in array technologies

Definitions

The term "Modified Vaccinia Ancara, (MVA)" defines according to the present invention a poxvirus or its deritatives, which derives from the MVA as described by Mayr, A , *et al* [1975], Infection 3, 6-14 Such a MVA is characterized by the well-known six major deletions and its extreme attenuation Beside tne diminished virulence or infectiosity such MVA has still an excellent

immunogenicity A typical MVA is strain MVA-575 that has been deposited at the European Collection of Animal Cell Cultures under the deposition number ECACC V00120707

Another preferred MVA is strain MVA-Vero or a derivative thereof The strain MVA-Vero has been deposited at the European Collection of Animal Cell Cultures under the deposition number ECACC 99101431 The safety of the MVA-Vero is reflected by biological, chemical and physical characteristics as described in the International Patent Application PCT/EP01/02703 In comparison to normal MVA, MVA-Vero has one additional genomic deletion

Still another preferred MVA is strain MVA-BN MVA-BN has been deposited at the European Collection of Animal Cell Cultures with the deposition number ECACC V00083008 MVA-BN virus is an extremely attenuated virus also derived from Modified Vaccinia Ankara virus

The term "derivatives" of a virus according to the present invention refers to progeny viruses showing the same characteristic features as the parent virus but showing differences in one or more parts of its genome The term "derivative of MVA" describes a virus, which has the same functional characteristics compared to MVA For example a derivative of MVA-BN has the characteristic features of MVA-BN One of these characteristics of MVA-BN or derivatives thereof is its attenuation and lack of replication in human HaCat cells

The term "Open reading frame" (ORF) defines a nucleotide sequence, which starts with a start codon and ends with a stop codon The nucleotide sequence of the ORF encodes for an amino acid sequence forming a peptide, polypeptide or a protein While most proteins or peptides encoded by the ORFs of MVA are essential for the viral life cycle,

there are also various ORFs, which code for proteins or peptides, which are non-essential for the viral life cycle ORFs in the MVA genome occur in two coding directions Consequently, the Polymerase activity occurs from 5'->3' or in other words left to right and, correspondingly, from right to left (5'<-3') It is common practice in poxvirology and it became a standard classification for Vaccinia viruses to identify ORF's by their orientation and their position on the different *Hind*III restriction digest fragments of the genome For the nomenclature the different *Hind*III fragments are named by descending capital letters corresponding with their descending size The ORF are numbered from left to right on each *Hind*III fragment and the orientation of the ORF is indicated by a capital L (standing for transcription from right to Left) or R (standing for transcription from left to Right) Additionally, there is a more recent publication of the MVA genome structure, which uses a different nomenclature, simply numbering the ORF from the left to the right end of the genome and indicating their orientation with a capital L or R (Antoine *et al* [1998], Virology 244, 365-396) As an example the I4L ORF, according to the old nomenclature, corresponds to the 065L ORF according to Antoine *et al* If not indicated differently, the present invention uses the nomenclature according to Antoine *et al*

The terms "upstream" and "downstream" indicate a position on a genomic DNA sequence In regard to orientation of the coding sequence and, thus, the direction of Polymerase activity, the term "upstream" is understood as earlier in the sequence Correspondingly, the term "downstream" refers to positions in the genomic sequence, which follow later in the sequence

The term "adjacent ORF" refers to the next following ORF located on the MVA genome, and can be understood as either in upstream or downstream direction of the ORF relative to the direction of polymerase activity

The term "intergenic region" (IR) refers to the nucleic acid sequence between two ORF of the MVA genome An intergenic region does not code for a protein, but may comprises regulatory elements, binding sites, promoter and/or enhancer sequences essential for transcriptional control of the gene expression of MVA gene products and, thereby, the regulatory control of the viral life cycle

The term "exogenous DNA sequences" is understood as a DNA sequence, which in nature is not normally found associated with the poxvirus of the invention The exogenous DNA sequence may comprise one or more cloning sites, one or more promoter or enhancer elements, one or more operably linked coding sequences, as well as one or more spacer sequences In general the spacer sequence is without own coding or regulatory activity and are located between several individual gene expression cassettes and/or at the left and/or the right end of the exogenous DNA sequence In case, that the exogenous DNA sequence is inserted in close distance to a start codon, the spacer sequence may comprise an alternative transcription control elements, which can control the transcription of the subsequent ORF According to the present invention the exogenous DNA sequence preferably comprises poxvirus specific transcription control elements, such as promoters or enhancers, which induces and controls transcription of the coding sequence or the coding sequences in the exogenous DNA

Summary of the invention

The invention *inter alia* comprises the following, alone or in combination

Recombinant Modified Vaccinia Ankara Virus (MVA) comprising one or more exogenous DNA sequences inserted into an intergenic region (IR) between two adjacent open reading frames (ORFs) of the viral genome,

MVA as above comprising exogenous DNA sequences inserted into two or more Irs,

MVA as above, wherein depending on the orientation of the ORFs the IR is flanked
(i) by the stop codons of the two adjacent ORFs,
(ii) by the start codon of the two adjacent ORFs,
(iii) by the stop codon of the first ORF and the start codon of the second ORF, or
(iv) by the start codon of the first ORF and the stop codon of the second ORF,

MVA as any above, whereby the exogenous DNA is inserted downstream of the stop codon of a first ORF and/or upstream of the start codon of a second ORF, wherein the first and the second ORF are adjacent ORFs,

MVA as any above, wherein the first ORF is selected from the group comprising the ORFs 005R, 006L, 007R, 008L, 019L, 020L, 021L, 023L, 028R, 029L, 037L, 045L, 050L, 052R, 054R, 055R, 056L, 057R, 058L, 062L, 065L, 069R, 070L, 081R, 082L, 086R, 088R, 089L, 092R, 093L, 107R, 108L, 122R, 123L, 125L, 126L, 135R, 136L, 137L, 141L, 148R, 149L, 152R, 153L, 156R, 157L, 159R, 160L, 165R, 166R, 167R, 169R, 170R, 176R, 180R, 184R, 185L, 187R, 188R and 191R,

MVA as any above, wherein the exogenous DNA is inserted upstream of the start codon of a second ORF, wherein the second ORF is selected from the group comprising the ORFs 007R, 028R, 090R, 095R and 154R,

MVA as any above, wherein the exogenous DNA sequence comprises at least one coding sequence under the transcriptional control of a poxviral transcription control element,

MVA as above, wherein the exogenous DNA sequence encodes one or more proteins, polypeptides, peptides, foreign antigens or antigenic epitopes,

MVA as any above, wherein the protein, polypeptide, peptide, antigen or the antigenic epitope is derived from Dengue virus, Japanese encephalitis virus, Hepatitis virus B, Hepatitis virus C and/or immunodeficiency viruses, preferable HIV,

MVA as any above, wherein the exogenous DNA sequence comprises a spacing sequence, which separates poxviral transcription control element and/or coding sequence in the exogenous DNA sequence from the stop codon and/or the start codon of the adjacent ORFs,

MVA as above, wherein the spacing sequence separating poxviral transcription control element and/or coding sequence in the exogenous DNA sequence from the stop codon of the adjacent ORF is one or more nucleotides,

MVA as above, wherein the spacing sequence separating poxviral transcription control element and/or coding sequence in the exogenous DNA Sequence from the start codon of the adjacent ORF is at least 30 nucleotides and optionally comprises a poxviral transcription control element,

MVA as any above as medicament and/or vaccine,

use of MVA as any above for the preparation of a medicament for the treatment of viral infections and/or proliferating diseases,

use as above for the treatment of dengue virus infection,

vaccine comprising the MVA as any above,

pharmaceutical composition comprising the MVA as any above and a pharmaceutically acceptable carrier, diluent, adjuvant and/or additive,

method for affecting, preferably inducing an immunological response in a living animal body including a human comprising administering the MVA as any above, the vaccine as above and/or the composition as above to the animal, including a human, in need thereof,

a plasmid vector comprising a DNA sequence derived from the genome of an MVA, wherein said DNA sequence comprises a complete or partial fragment of an IR between two adjacent ORF of the viral genome and inserted into said IR-derived sequence a poxviral transcription control element with at least one cloning site for the insertion of the exogenous DNA and optional a reporter- and/or selection gene cassette,

a plasmid vector as above, wherein the IR is adjacent to an ORF selected from the group comprising the ORFs 005R, 006L, 007R, 008L, 019L, 020L, 021L, 023L, 028R, 029L, 037L, 045L, 050L, 052R, 054R, 055R, 056L, 057R, 058L, 062L, 065L, 069R, 070L, 081R, 082L, 086R, 088R, 089L, 090R, 092R, 093L, 095R, 107R, 108L, 122R, 123L, 125L, 126L, 135R, 136L, 137L, 141L, 148R, 149L, 152R, 153L, 154R, 156R, 157L, 159R, 160L, 165R, 166R, 167R, 169R, 170R, 176R, 180R, 184R, 185L, 187R, 188R and 191R,

a method for producing a MVA as any above comprising the steps of

(i) transfecting cells a plasmid vector as any above comprising a complete or partial fragment of an IR between two adjacent ORF of the MVA genome and inserted into said IR-derived sequence a poxviral transcription control element with at least one cloning site for the insertion of the exogenous DNA and optional a reporter- and/or selection gene cassette,

(ii) infecting the transfected cells from (i) with a MVA,

(iii) identifying, isolating and optionally purifying a MVA as any above, comprising one or more exogenous DNA sequences inserted into an intergenic region (IR) between two adjacent open reading frames (ORFs) of the viral genome,

DNA sequence or part thereof derived from or homologous to the MVA as any above, wherein the DNA sequence comprises fragments of the genome of the MVA as any above, comprising a complete or partial fragment of an IR between two adjacent ORF of the viral genome and comprising a DNA sequence inserted into said IR,

DNA sequence as above, wherein the IR is adjacent to an ORF selected from the group comprising the ORFs 005R, 006L, 007R, 008L, 019L, 020L, 021L, 023L, 028R, 029L, 037L, 045L, 050L, 052R, 054R, 055R, 056L, 057R, 058L, 062L, 065L, 069R, 070L, 081R, 082L, 086R, 088R, 089L, 090R, 092R, 093L, 095R, 107R, 108L, 122R, 123L, 125L, 126L, 135R, 136L, 137L, 141L, 148R, 149L, 152R, 153L, 154R, 156R, 157L, 159R, 160L, 165R, 166R, 167R, 169R, 170R, 176R, 180R, 184R, 185L, 187R, 188R and 191R,

Use of the DNA sequence as any above for detecting cells or individuals infected with a MVA as any above and/or identifying a MVA as any above

Short description of the Figures

Figure 1: Restriction map of the vector construct, pBNX39, comprising about 600bp of MVA sequences flanking the insertion site after the I4L ORF The plasmid additionally comprises exogenous DNA (Ecogpt under the transcriptional control a poxvirus promoter, P) between the flanking sequences Flank 1 (F1 I4L) and Flank 2 (F2 I4L) F1rpt stands for a repetitive sequence of Flank 1 to allow deletion of the reporter cassette from a resulting recombinant virus . Further abbreviations AmpR = Ampicilin resistance gene, bps = base pairs

Figure 2 und 3: Restriction map of the vector construct, pBNX51 and pBNX67, comprising about 600bp of MVA sequences flanking the insertion site after the ORF 137L (Flank 1 F1A137L corresponds to position 129340 - 129930 of the MVA genome, Flank 2 F2A137L corresponds to position 129931 - 130540 of the MVA genome) Additionally the vector pBNX67 comprises exogenous DNA (NPT II gene (neomycin resistance) under the transcriptional control of a poxvirus promoter, P) between the flanking sequences F2rpt stands for a repetitive sequence of Flank 2 to allow deletion of the reporter cassette from a resulting recombinant virus Further abbreviations AmpR = Ampicilin resistance gene, bps = base pairs, IRES = internal ribosomal entry site, EGFP = gene for the enhanced green fluorescent protein

Figure 4: Restriction map of the vector construct, pBNX79, comprising about 600 bps of MVA sequences

flanking the insertion site between the ORF 007R and 008L (Flank 1 F1IGR07/08 starts at position 12200 of the MVA genome, Flank 2 F2IGR07/08 stops at position 13400 of the MVA genome) F2rpt stands for a repetitive sequence of Flank 2 to allow deletion of the reporter cassette from a resulting recombinant virus Further abbreviations AmpR = Ampicilin resistance gene, bps = base pairs

Figure 5: Restriction map of the vector construct, pBNX80, comprising about 600/640 bps of MVA sequences flanking the insertion site between the ORF 044L and 045L (Flank 1 F1IGR44/45 starts at position 36730 of the MVA genome, Flank 2 F2IGR44/45 stops at position 37970 of the MVA genome) F2rpt stands for a repetitive sequence of Flank 2 to allow deletion of the reporter cassette from a resulting recombinant virus Further abbreviations AmpR = Ampicilin resistance gene, bps = base pairs

Figure 6: Restriction map of the vector construct, pBNX90, comprising about 596/604 bps of MVA sequences flanking the insertion site between the ORF 148R and 149L (Flank 1 F1IGR148/149 starts at position 136900 of the MVA genome, Flank 2 F2IGR148/149 stops at position 138100 of the MVA genome) F2rpt stands for a repetitive sequence of Flank 2 to allow deletion of the reporter cassette from a resulting recombinant virus Further abbreviations AmpR = Ampicilin resistance gene, bps = base pairs

Figure 7: Schematic insertion site after the I4L ORF The intergeneic region of MVA (Genbank Ac U94848) in lower case lettering (except for the putative early promoter sequence for the I3L ORF) between the I3L ORF (MVA064L)

and I4L ORF (MVA065L) Insertion point by homologous recombination is by substitution of the T/A marked off in bold

The following examples will further illustrate the present invention It will be well understood by any person skilled in the art that the provided examples in no way are to be interpreted in a way that limits the present invention to these examples The scope of the invention is only to be limited by the full scope of the appended claims

Example 1

Insertion vector pBNX39

For the insertion of exogenous sequences into the intergenetic region adjacent to the 065L ORF (insertion site is at genome position 56760) of MVA, a vector was constructed, which comprises about 1200 bp of the flanking sequences adjacent to the insertion site These flanking sequence are separated into two flanks comprising on one flank about 610 bp of the 065L ORF (alternative nomenclature I4L ORF) and on the other part about 580 bp of the intergenic region behind the 065L ORF as well as parts of the proximate ORF In between these flanking sequences is located an *Ecogpt* gene (*gpt* stands for phosphoribosyltransferase gene isolated from *E coli*) under the transcriptional control of a poxviral promoter Additionally, there is at least one cloning site for the insertion of additional genes or sequences to be inserted into the intergenetic region behind the I4L ORF An exemplary vector construct according to the present invention is disclosed in Figure 1 (pBNX39)

Generation of the recombinant MVA via homologous recombination

Foreign genes can be inserted into the MVA genome by homologous recombination For that purpose the foreign gene of interest is cloned into a plasmid vector, as described above This vector is transfected in MVA infected cells The recombination takes place in the cytoplasm of the infected and transfected cells With help of the selection and/or reporter cassette, which is also contained in the insertion vector, cells comprising recombinant viruses are identified and isolated

For homologous recombination BHK (Baby hamster kidney) cells or CEF (primary chicken embryo fibroblasts) are seeded in 6 well plates using DMEM (Dulbecco's Modified Eagles Medium, Gibco BRL) + 10% fetal calf serum (FCS) or VP-SFM (Gibco BRL) + 4mmol/l L-Glutamine for a serum free production process

Cells need to be still in the growing phase and therefore should reach 60-80% confluence on the day of transfection Cells were counted before seeding, as the number of cells has to be known for determination of the multiplicity of infection (moi) for infection

For the infection the MVA stock is diluted in DMEM/FCS or VP-SFM/L-Glutamine so that 500 µl dilution contain an appropriate amount of virus that will give a moi of 0 01 Cells are assumed to have divided once after seeding The medium is removed from cells and cells are infected with 500µl of diluted virus for 1 hour rocking at room temperature The inoculum is removed and cells are washed with DMEM/VP-SFM Infected cells are left in 1 6ml DMEM/FCS and VP-SFM/L- Glutamine respectively while setting up the transfection reaction (Qiagen Effectene Kit)

For the transfection the "Effectene" transfection kit (Qiagen) is used A transfection mix is prepared of 1-2 µg of linearized insertion vector (total amount for multiple transfection) with buffer EC to give a final volume of 100 µl Add 3 2 µl Enhancer, vortex and incubate at room temperature for 5 min Then, 10 µl of Effectene are added after vortexing stock tube and the solution is mixed thoroughly by vortexing and incubated at room temperature for 10 min 600 µl of DMEM/FCS and VP-SFM/L-Glutamine respectively, are added, mixed and

subsequently, the whole transfection mix is added to the cells, which are already covered with medium Gently the dish is rocked to mix the transfection reaction Incubation takes place at 37°C with 5%CO_2 over night The next day the medium is removed and replaced with fresh DMEM/FCS or VP-SFM/L-Glutamine Incubation is continued until day 3

For harvesting the cells are scraped into medium, then the cell suspension is transferred to an adequate tube and frozen at -20°C for short-term storage or at -80°C for long term storage

Insertion of Ecogpt in the I4L insertion site of MVA

In a first round, cells were infected with MVA according to the above-described protocol and were additionally transfected with insertion vector pBNx39 containing the *Ecogpt* gene (*Ecogpt* or shortened to *gpt* stands for phosphoribosyltransferase gene) as reporter gene Resulting recombinant viruses were purified by 3 rounds of plaque purification under phosphribosyl-transferase metabolism selection by addition of mycophenolc acid, xanthin and hypoxanthin Mycophenolic acid (MPA) inhibits inosine monophosphate dehydrogenase and results in blockage of purine synthesis and inhibition of viral replication in most cell lines This blockage can be overcome by expressing *Ecogpt* from a constitutive promoter and providing the substrates xanthine and hypoxanthine

Resulting recombinant viruses were identified by standard PCR assays using a primer pair selectively amplifying the expected insertion site To amplify the I4L insertion side primer pair, BN499 (CAA CTC TCT TCT TGA TTA CC, SEQ ID NO 1) and BN500 (CGA TCA AAG TCA ATC TAT G, SEQ ID

NO 2) were used In case the DNA of the empty vector virus MVA is amplified the expected PCR fragment is 328 nucleotides (nt) long, in case a recombinant MVA is amplified, which has incorporated exogenous DNA at the I4L insertion site, the fragment is correspondingly enlarged

Example 2

Insertion vector pBNX67

The MVA sequences adjacent the new insertion site (at genome position 129940) after the ORF 137L were isolated by standard PCR amplification of the sequence of interest using the following primers

oBN543 (TCCCCGCGGAGAGGCGTAAAAGTTAAATTAGAT, SEQ ID NO 3) and oBN544 (TGATCTAGAATCGCTCGTAAAAACTGCGGAGGT, SEQ ID NO 4) for isolating Flank 1,
oBN578 (CCGCTCGAGTTCACGTTCAGCCTTCATGC, SEQ ID NO 5) and oBN579 (CGGGGGCCCTATTTTGTATAATATCTGGTAAG, SEQ ID NO 6) for isolating Flank 2

The PCR fragment comprising Flank 1 was treated with the restriction enzymes SacII and XbaI and ligated to a SacII/XbaI digested and dephosphorylated basic vector, such as pBluescript (Stratagene)

The resulting plasmid was XhoI/ApaI digested, dephosphorylated and ligated to the XhoI/ApaI digested PCR fragment comprising Flank 2

Optionally a repetitive sequence of Flank 2, which had been isolated by PCR using the primers oBN545 (CGGCTGCAGGGTACCTTCACGTTCAGCCTTCATGC, SEQ ID NO 7) and oBN546 (CGGAAGCTTTATATGGTTTAGGATATTCTGTTTT, SEQ ID NO 8) and which became HindIII/PstI digested, was

inserted into the HindIII/PstI site of the resulting vector Figure 3 shows the vector (pBNX51)

A reporter cassette comprising a synthetic promoter, NPT II gene (neomycin resistance), poly-A region, IRES, EGFP gene (Ps-NPTII-polyA-IRES-EGFP) was Ecl136II/XhoI digested and inserted into the HindIII/XhoI site of the insertion vector, wherein the HindIII site was blunt ended with T4 DNA Polymerase (Roche) A restriction map of an exemplary vector construct according to this example is disclosed in Figure 3 (pBNX67)

The vector can be used to generate a recombinant MVA - following the above-mentioned protocol - carrying an exogenous sequence in the intergenic region between two adjacent ORFs

Example 3

Insertion vector pBNX79

The MVA sequences adjacent the new insertion site (at genome position 12800) between the ORF 007R and 008L were isolated by standard PCR amplification of the sequence of interest using the following primers

IGR 07/08 F1up
(CGCGAGCTCAATAAAAAAAAGTTTTAC, SEQ ID NO 9) and IGR 07/08 F1end (AGGCCGCGGATGCATGTTATGCAAAATAT, SEQ ID NO 10) for isolating Flank 1,
IGR 07/08 F2up
(CCGCTCGAGCGCGGATCCCAATATATGGCATAGAAC, SEQ ID NO 11) and IGR 07/08 F2end
(CAGGGCCCTCTCATCGCTTTCATG, SEQ ID NO 12) for isolating Flank 2

The PCR fragment comprising Flank 1 was treated with the restriction enzymes SacII and SacI and ligated to a SacII/SacI digested and dephosphorylated basic vector, such as pBluescript (Stratagene)

The resulting plasmid was XhoI/ApaI digested, dephosphorylated and ligated to the XhoI/ApaI digested PCR fragment comprising Flank 2

Optionally a repetitive sequence of Flank 2, which had been isolated by PCR using the primers IGR 07/08 F2up (CCGCTCGAGCGCGGATCCCAATATATGGCATAGAAC, SEQ ID NO 11) and IGR 07/08 F2mid (TTTCTGCAGTGATATTTATCCAATACTA, SEQ ID NO 13) and which is BamHI/PstI digested, was inserted into the BamHI/PstI site of the resulting vector

Any reporter or therapeutical gene comprising cassette, having e g a poxviral promoter, a marker gene, a poly-A region and optionally an IRES element, a further gene, e g expressing a therapeutically active substance or gene product, can be blunt ended with T4 DNA Polymerase (Roche) after an restriction digest and inserted into a suitable cloning site of the plasmid vector Considering a reporter gene cassette the HindIII, XhoI or PstI restriction enzyme site between Flank 2 and the Flank-2-repitition is preferred as cloning site Considering an expression unite for a therapeutic gene, comprising a therapeutic gene and an operably linked promoter, this expression unit is inserted into the PacI site

A restriction map of an exemplary vector construct according to this example is disclosed in Figure 4 (pBNX79)

The vector can be used to generate a recombinant MVA - following the above-mentioned protocol - carrying an

exogenous sequence in the intergenic region between two adjacent ORFs

Example 4

Insertion vector pBNX80

The MVA sequences adjacent the new insertion site (at genome position 37330) between the ORF 044L and 045L were isolated by standard PCR amplification of the sequence of interest using the following primers

IGR44/45F1up (CGCGAGCTCATTTCTTAGCTAGAGTGATA, SEQ ID NO 14) and IGR44/45F1end (AGGCCGCGGAGTGAAAGCTAGAGAGGG, SEQ ID NO 15) for isolating Flank 1, IGR44/45F2up (CCGCTCGAGCGCGGATCCTAAACTGTATCGATTATT, SEQ ID NO 16) and IGR44/45F2end (CAGGGCCCCTAAATGCGCTTCTCAAT, SEQ ID NO 17) for isolating Flank 2

The PCR fragment comprising Flank 1 was treated with the restriction enzymes SacII and SacI and ligated to a SacII/SacI digested and dephosphorylated basic vector, such as pBluescript (Stratagene)

The resulting plasmid was XhoI/ApaI digested, dephosphorylated and ligated to the XhoI/ApaI digested PCR fragment comprising Flank 2

Optionally a repetitive sequence of Flank 2, which had been isolated by PCR using the primers IGR44/45F2up (CCGCTCGAGCGCGGATCCTAAACTGTATCGATTATT, SEQ ID NO 16) and IGR44/45F2mid (TTTCTGCAGCCTTCCTGGGTTTGTATTAACG, SEQ ID NO 18) and which became BamHI/PstI digested, was inserted into the BamHI/PstI site of the resulting vector

Any reporter or therapeutical gene comprising cassette, having e g a poxviral promoter, a marker gene, a poly-A region and optionally an IRES element, a further gene, e g expressing a therapeutically active substance or gene product, can be blunt ended with T4 DNA Polymerase (Roche) after an restriction digest and inserted into a suitable cloning site of the plasmid vector Considering a reporter gene cassette the HindIII, XhoI or PstI restriction enzyme site between Flank 2 and the Flank-2-repitition is preferred as cloning site Considering an expression unite for a therapeutic gene, comprising a therapeutic gene and an operably linked promoter, this expression unite is inserted into the PacI site

A restriction map of an exemplary vector construct according to this example is disclosed in Figure 5(pBNX80)

The vector can be used to generate a recombinant MVA - following the above-mentioned protocol - carrying an exogenous sequence in the intergenic region between two adjacent ORFs

Example 5

Insertion vector pBNX90

The MVA sequences adjacent the new insertion site (at genome position 137496) between the ORF 148R and 149L were isolated by standard PCR amplification of the sequence of interest using the following primers

IGR148/149F1up (TCCCCGCGGGGACTCATAGATTATCGACG, SEQ ID NO 19) and IGR148/149F1end (CTAGTCTAGACTAGTCTATTAATCCACAGAAATAC, SEQ ID NO 20) for isolating Flank 1,

IGR148/149F2up (CCCAAGCTTGGGCGGGATCCCGTTTCTAGTATGGGGATC, SEQ ID NO 21) and IGR148/149F2end (TAGGGCCCGTTATTGCCATGATAGAG, SEQ ID NO 22) for isolating Flank 2

The PCR fragment comprising Flank 1 was treated with the restriction enzymes SacII and XbaI and ligated to a SacII/XbaI digested and dephosphorylated basic vector, such as pBluescript (Stratagene)

The resulting plasmid was HindIII/ApaI digested, dephosphorylated and ligated to the HindIII/ApaI digested PCR fragment comprising Flank 2

Optionally a repetitive sequence of Flank 2, which had been isolated by PCR using the primers IGR148/149F2up (CCCAAGCTTGGGCGGGATCCCGTTTCTAGTATGGGGATC, SEQ ID NO 21) and IGR148/149F2mid (TTTCTGCAGTGTATAATACCACGAGC, SEQ ID NO 23) and which became BamHI/PstI digested, was inserted into the BamHI/PstI site of the resulting vector

Any reporter or therapeutical gene comprising cassette, having e g a poxviral promoter, a marker gene, a poly-A region and optionally an IRES element, a further gene, e g expressing a therapeutically active substance or gene product, can be blunt ended with T4 DNA Polymerase (Roche) after an restriction digest and inserted into a suitable cloning site of the plasmid vector Considering a reporter gene cassette the HindIII, XhoI or PstI restriction enzyme site between Flank 2 and the Flank-2-repitition is preferred as cloning site Considering an expression unite for a therapeutic gene, comprising a therapeutic gene and an operably linked promoter, this expression unite is inserted into the PacI site

A restriction map of an exemplary vector construct according to this example is disclosed in Figure 6 (pBNX90)

The vector can be used to generate a recombinant MVA - following the above-mentioned protocol - carrying an exogenous sequence in the intergenic region between two adjacent ORFs

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Claims

(1) Recombinant Modified Vaccinia Ankara Virus (MVA) comprising one or more exogenous DNA sequences inserted into an intergenic region (IR) between two adjacent open reading frames (ORFs) of the viral genome

(2) MVA according to claim 1 comprising exogenous DNA sequences inserted into two or more IRs

(3) MVA according to the claims 1 or 2, whereby the exogenous DNA is inserted downstream of the stop codon of a first ORF and/or upstream of the start codon of a second ORF, wherein the first and the second ORF are adjacent ORFs

(4) MVA according to claim 3, wherein the first ORF is selected from the group comprising the ORFs 005R, 006L, 007R, 008L, 019L, 020L, 021L, 023L, 028R, 029L, 037L, 045L, 050L, 052R, 054R, 055R, 056L, 057R, 058L, 062L, 065L, 069R, 070L, 081R, 082L, 086R, 088R, 089L, 092R, 093L, 107R, 108L, 122R, 123L, 125L, 126L, 135R, 136L, 137L, 141L, 148R, 149L, 152R, 153L, 156R, 157L, 159R, 160L, 165R, 166R, 167R, 169R, 170R, 176R, 180R, 184R, 185L, 187R, 188R and 191R

(5) MVA according to claim 3, wherein the exogenous DNA is inserted upstream of the start codon of a second ORF, wherein the second ORF is selected from the group comprising the ORFs 007R, 028R, 090R, 095R and 154R

(6) MVA according to any of the claims 1 to 5, wherein the exogenous DNA sequence comprises at least one coding sequence under the transcriptional control of a poxviral transcription control element

(7) MVA according to any of the claims 1 to 6, wherein the protein, polypeptide, peptide, antigen or the antigenic epitope is derived from Dengue virus, Japanese encephalitis virus, Hepatitis virus B, Hepatitis virus C and/or immunodeficiency viruses, preferable HIV

(8) MVA according to any of the claims 1 to 7 as medicament and/or vaccine

(9) Use of MVA according to any of the claims 1 to 7 for the preparation of a medicament for the treatment or prophylaxis of viral infections and/or proliferating diseases

(10) Vaccine comprising the MVA according to any of the claims 1 to 7

Abstract

The present invention relates to recombinant Modified Vaccinia Ankara (MVA) viruses and, particularly, to novel insertion sites useful for the integration of exogenous sequences into the MVA genome The present invention further provides plasmid vectors to insert exogenous DNA into the genome of MVA Furthermore, the present invention provides recombinant MVA comprising exogenous DNA at said new insertion sites as medicine or vaccine

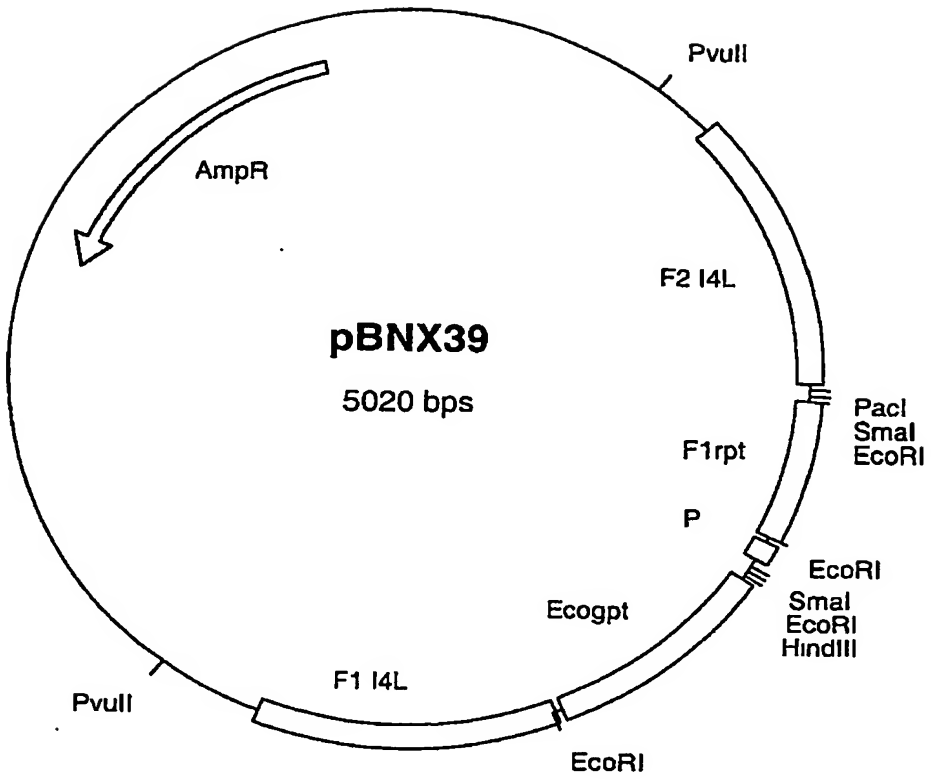


Fig 1

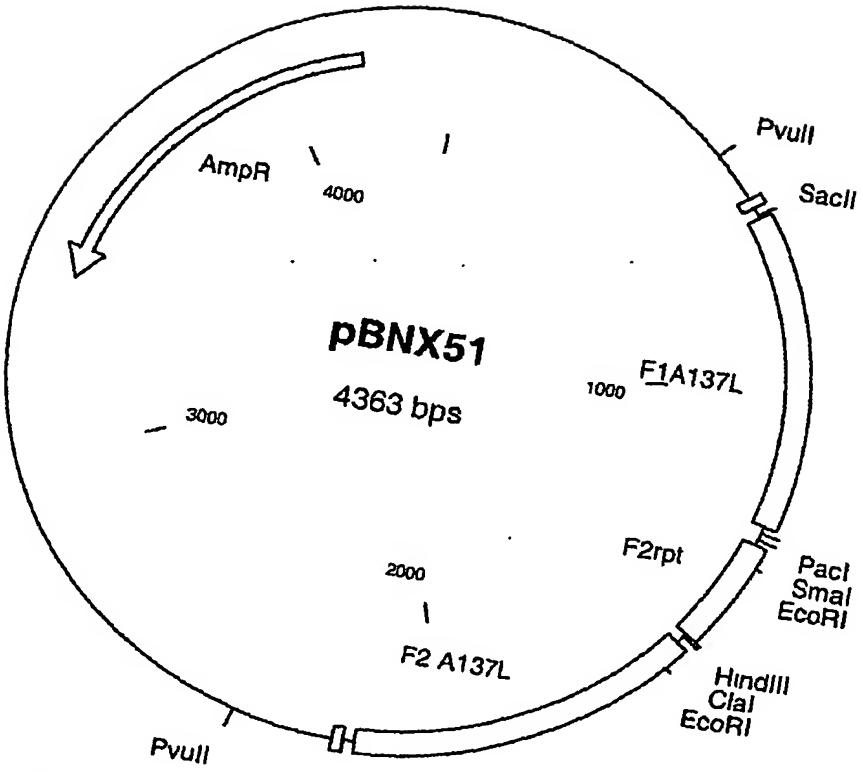


Fig 2

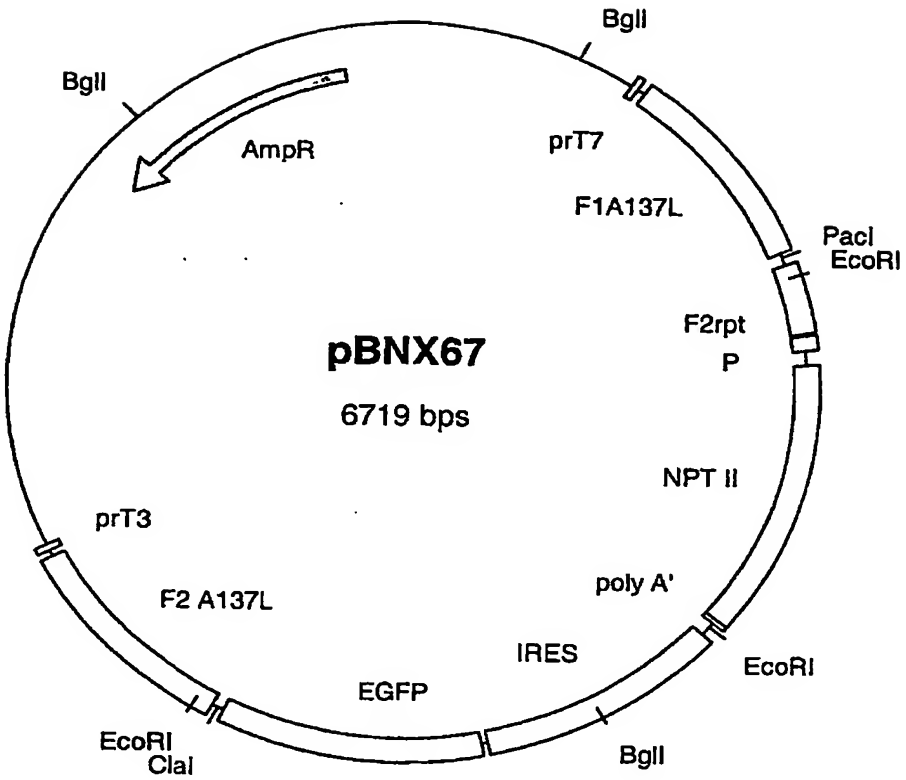


Fig 3

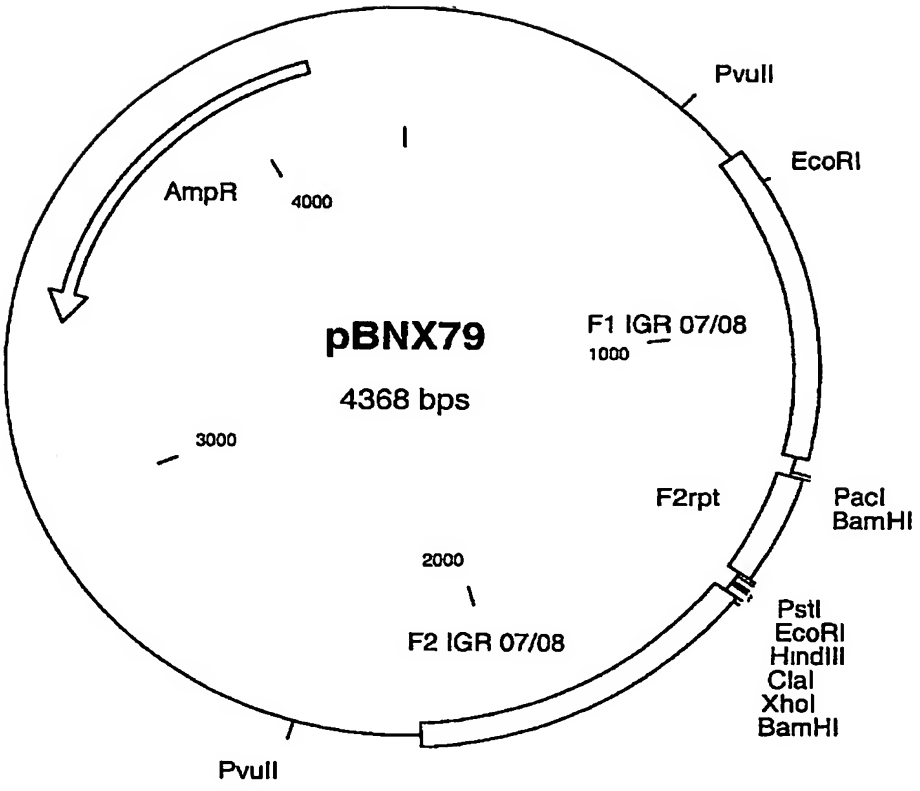


Fig 4

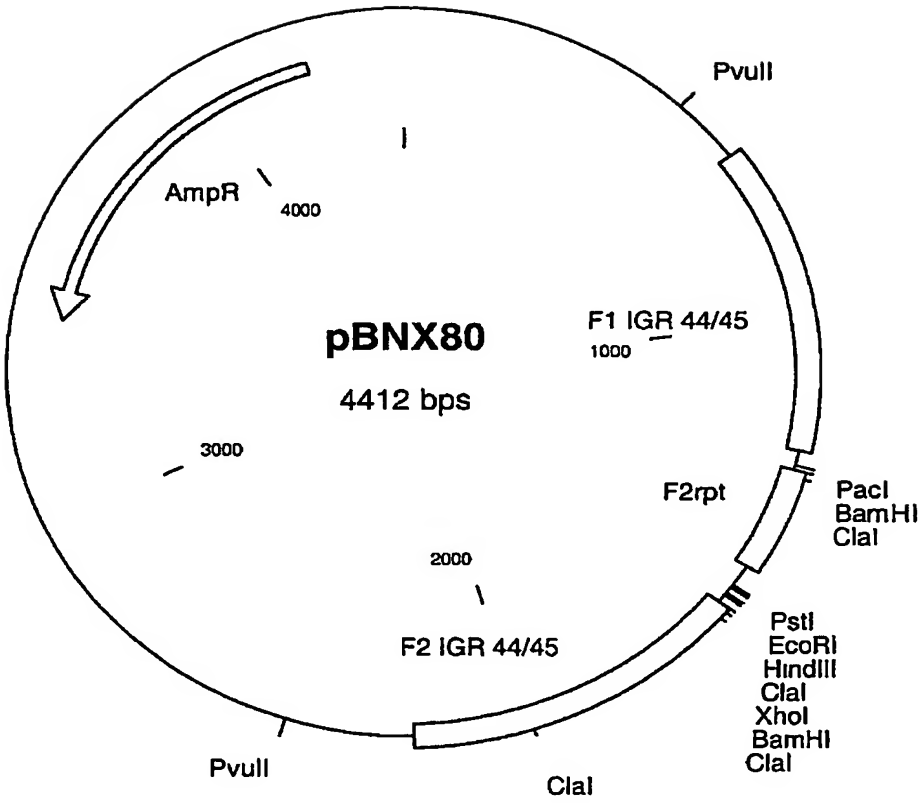


Fig 5

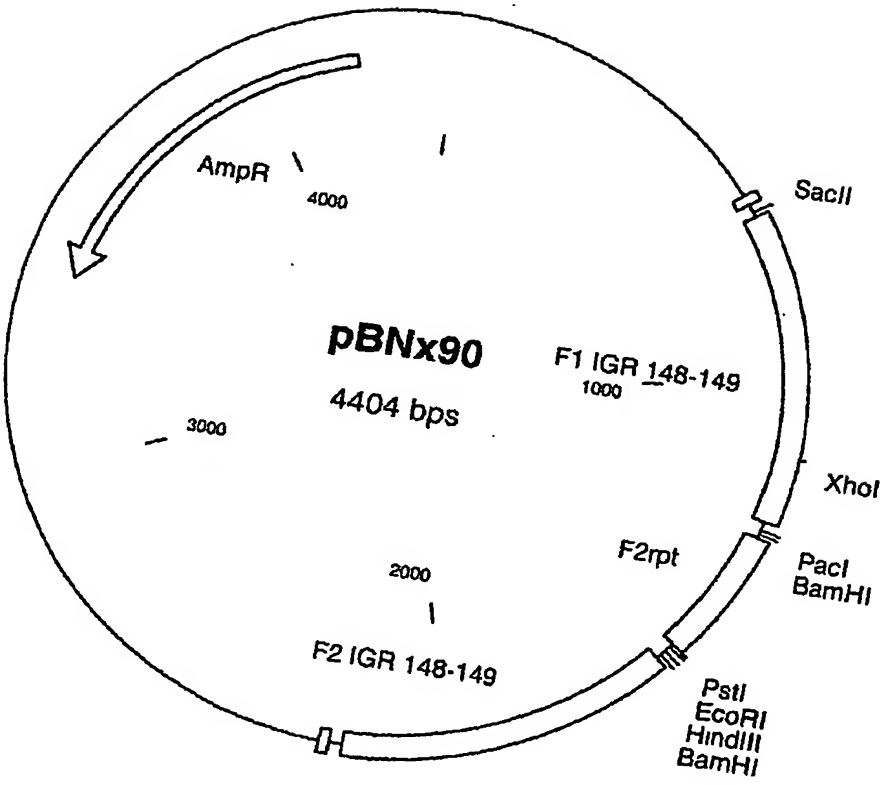
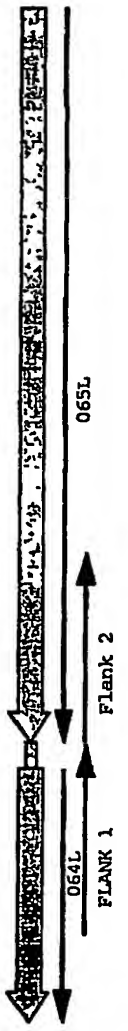


Fig 6



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CAGAACCACTTCAAAGTTGAGAGAAGAACTAATGGAATGAGTACtaatttggatttattaacatgaaacattatatt

<----- I3L ORF

56818 tgatatATATTTTTCACTTTATCttcatttgagaataaaaatgTttttgtTTAACCACTGCATGATGTACAGATTTCGG
actataTATAAAAGTGAAATAgagtaaactctttatttttacAaaaacaAATTGGTGACGTACTACATGTCTAAAGCC

Putative early promoter element Insertion Point <------ I4L ORF

56895 AATCGCAAACCACCAGTGGTTTTATTTTATCCTTGTCCAATGTGAATTGAATGGGAGCGGATGCGGGTTTCGTACGT
TTAGCGTTTGGTGGTCACCAAAATAAAATAGGAACAGGTTACACTTAACTTACCCTCGCCTACGCCCAAAGCATGCA

Fig 7

Oplysning om deponering af biologisk materiale

Ansøgningen omfatter følgende deponeringer i henhold til Patentlovens § 8a, stk 1 eller Brugsmodellovens § 8, stk 1

Patent- og
Varemærkestyrelsen
Erhvervsministeriet

Helgeshøj Allé 81
2630 Taastrup

Tlf 43 50 80 00
Fax 43 50 80 01
Postgiro 8 989 923
E post pvs@dkpto dk
www dkpto dk

A Identifikation af deponeringer

1 Vedrørende det på side 16 linie 12-16 i beskrivelsen omtalte biologiske materiale

Deponeringsinstitutionens navn
European Collection of Cell Cultures, CAMR

Deponeringsinstitutionens adresse (inklusive postnummer og land)
Salisbury, Wiltshire SP4 OJG, United Kingdom, Tel + 44 19 80 61 25 12

Dato for deponering 30 August 2000 Løbenummer V00083008

2 Vedrørende det på side 16 linie 1-3 i beskrivelsen omtalte biologiske materiale

Deponeringsinstitutionens navn
European Collection of Cell Cultures, CAMR

Deponeringsinstitutionens adresse (inklusive postnummer og land)
Salisbury, Wiltshire SP4 OJG, United Kingdom, Tel + 44 19 80 61 25 12

Dato for deponering 7 Dezember 2000 Løbenummer V00120707

3 Vedrørende det på side 16 linie 4-11 i beskrivelsen omtalte biologiske materiale

Deponeringsinstitutionens navn
European Collection of Cell Cultures, CAMR

Deponeringsinstitutionens adresse (inklusive postnummer og land)
Salisbury, Wiltshire SP4 OJG, United Kingdom, Tel + 44 19 80 61 25 12

Dato for deponering 14 Oktober 1999 Løbenummer V99101431

☐ Yderligere oplysninger på et følgende ark

B Yderligere angivelser, fx om det biologiske materiales farlighed, geografisk oprindelse

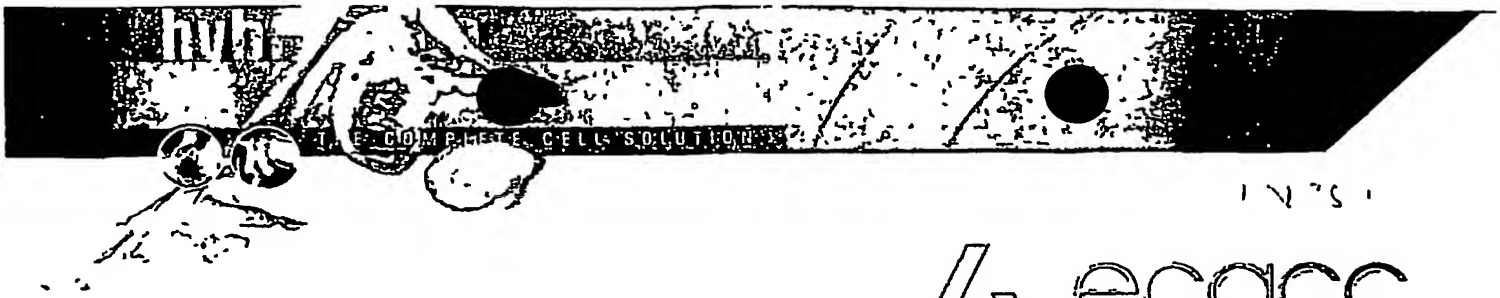
☑ Oplysningerne fortsættes på et vedføjet ark

C ☑ Det begæres at udlevering af en prøve i tiden indtil ansøgningen er fremlagt eller endeligt afgjort uden at være fremlagt kun sker til særlig sagkyndig jfr PL § 22 stk 7 eller BML § 8 stk 2

Dato og underskrift

(Peter Wulff)

17 mar00/s



ecacc
European Collection
of Cell Cultures

Centre for Applied Microbiology and Research
&
European Collection of Cell Cultures

This document certifies that Virus
(Deposit Ref V00083008) has been accepted as a patent deposit,
in accordance with
The Budapest Treaty of 1977,
with the European Collection of Cell Cultures on 30TH August 2000

Dr P J Packer
Quality Manager, ECACC

European Collection of Cell Cultures CAMR Salisbury Wiltshire SP4 0JG UK
Tel 44 (0) 1980 612512 Fax 44 (0) 1980 611315 Email ecacc@camr.org.uk Web Site ecacc.org.uk

IV CONDITIONS UNDER WHICH THE VIABILITY TEST HAS BEEN PERFORMED [4]	
V00083008 - MVA-BN VIABILITY OF MVA-BN WAS TESTED BY GROWING THE VIRUS ON BHK CELLS AND CALCULATING THE TCD50	
V INTERNATIONAL DEPOSITARY AUTHORITY	
Name Dr P J Packer ECACC CAMR Address Porton Down Salisbury Wiltshire SP4 0JG	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s) Date 14/12/00 PJPacker

4 Fill in if the information has been requested and if the results of the test were negative

BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

TO

BAVARIAN NORDIC RESEARCH
INSTITUTE GMBH
FRAUNHOFERSTRASSE 18B
D-82152 MARTINSRIED
GERMANY

VIABILITY STATEMENT
Issued pursant to Rule 10 2 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified on the following page

NAME AND ADDRESS OF THE PARTY
TO WHOM THE VIABILITY OF STATEMENT
IS ISSUED

I DEPOSITOR	II IDENTIFICATION OF THE MICROORGANISM
Name BAVARIAN NORDIC RESEARCH INSTITUTE GMBH Address FRAUNHOFERSTRASSE 18B D-82152 MARTINSRIED GERMANY	Accession number given by the INTERNATIONAL DEPOSITORY AUTHORITY V00083008 Date of the deposit or of the transfer 30TH August 2000

II VIABILITY STATEMENT

The viability of the microorganism identified under II above was tested on [2] On that date, the said microorganism was

[X] [3] viable

[ ] [3] no longer viable

1 Indicate the date of the original deposit or, where a new deposit or a transfer has been made, the most relevant date (date of the new deposit or date of the transfer)

2 In the cases referred to in Rule 10 2 (a) (ii) and (iii), refer to the most recent viability test

3 Mark with a cross the applicable box

BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

TO

BAVARIAN NORDIC RESEARCH
INSTITUTE GMBH
FRAUNHOFERSTRASSE 18B
D-82152 MARTINSRIED
GERMANY

NAME AND ADDRESS
OF DEPOSITOR

I IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY
MVA-BN	V00083008

II SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

The microorganism identified under I above was accompanied by

[X] A scientific description

[ ] A proposed taxonomic designation

(Mark with a cross where applicable)

III RECEIPT AND ACCEPTANCE

This International Depository Authority accepts the microorganism identified under I above, which was received by it on 30TH August 2000 (date of the original deposit)[1]

IV RECEIPT OF REQUEST FOR CONVERSION

The microorganism identified under I above was received by this International Depository Authority on (date of the original deposit) and
A request to convert the original deposit to a deposit under the Budapest Treaty was received by it on (date of receipt of request for conversion)

IV INTERNATIONAL DEPOSITORY AUTHORITY

Name Dr P J Packer

Address ECACC
CAMR
Porton Down
Salisbury SP4 0JG

Signature(s) of person(s) having the power to represent the International Depository Authority or of authorized officials(s)

Date PS Packer 14/12/00

1 Where Rule 6 4(d) applies, such date is the date on which the status of international depositary authority was acquired

Form BP/4 (sole page)

1991

Certificate of Analysis

Product Description MVA-BN
Accession Number 00083008

Test Description The Detection of Mycoplasma by Isolation on Mycoplasma Pig Serum Agar and in Mycoplasma Horse Serum Broth
SOP QC/MYCO/01/02

Acceptance Criterion/Specification: All positive controls (*M pneumoniae & M orale*) must show evidence of mycoplasma by typical colony formation on agar plates Broths are subcultured onto Mycoplasma Pig Serum Agar where evidence of mycoplasma by typical colony formation is evaluated All negative control agar plates must show no evidence of microbial growth
The criteria for a positive test result is evidence of mycoplasma by typical colony formation on agar A negative result will show no such evidence

Test Number 21487

Date. 27/11/00

Result.

Positive Control	Positive
Negative Control	Negative
Test Result	Negative
Overall Result	PASS

Test Description: Detection of Mycoplasma using a Vero indicator cell line and Hoechst 33258 fluorescent detection system
SOP QC/MYCO/07/05

Acceptance Criterion/Specification: The Vero cells in the negative control are clearly seen as fluorescing nuclei with no cytoplasmic fluorescence Positive control (*M orale*) must show evidence of mycoplasma as fluorescing nuclei plus extra nuclear fluorescence of mycoplasma DNA Positive test results appear as extra nuclear fluorescence of mycoplasma DNA Negative results show no cytoplasmic fluorescence

Test Number. 21487

Date 27/11/00

Result:

Positive Control	Positive
Negative Control	Negative
Test Result	Negative
Overall Result	PASS

Authorised by ..... **.. ECACC, Head of Quality** 4/12/00 **Date**

Certificate of Analysis

Product Description MVA-BN
Accession Number 00083008

Test Description Detection of bacteria and fungi by isolation on Tryptone Soya Broth (TSB) and in Fluid Thioglycollate Medium (FTGM) SOP QC/BF/01/02

Acceptance Criterion/Specification All positive controls (*Bacillis subtilus, Clostridium sporogenes* and *Candida albicans*) show evidence of microbial growth (turbidity) and the negative controls show no evidence of microbial growth (clear)
The criteria for a positive test is turbidity in any of the test broths All broths should be clear for negative test result

Test Number 21487
Date 27/11/00
Result

Positive Control	Positive
Negative Control	Negative
Test Result	Negative
Overall Result	PASS

Test Description. Determination of $TCID_{50}$ of cytopathic Virus titration (SOP ECACC/055) Cell

Acceptance Criterion/Specification/Criteria Negative controls should show no sign of Cytopathic effects The Test Sample is serially diluted into in 4 wells of indicator cell lines for each dilution Cytopathic effects indicate that virus is present Virus titre is calculated using the below equation where x is the value obtained from a standard $TCID_{50}$ Table as a result of the distribution of the wells displaying less than 4 positive wells per dilution, and y is the value of the highest dilution where all 4 wells are positive

$$TCID_{50} = \frac{1}{y} \times 10^{(1+x)}$$

Date 01/12/00

Result

Indicator Cell Line	BHK21 (Clone 13)
Negative Control	NO CPE
Test Sample	CPE
Distribution of less that 4 positive wells	4, 4, 4, 3, 0
X	1.25
Y	10^{-3}

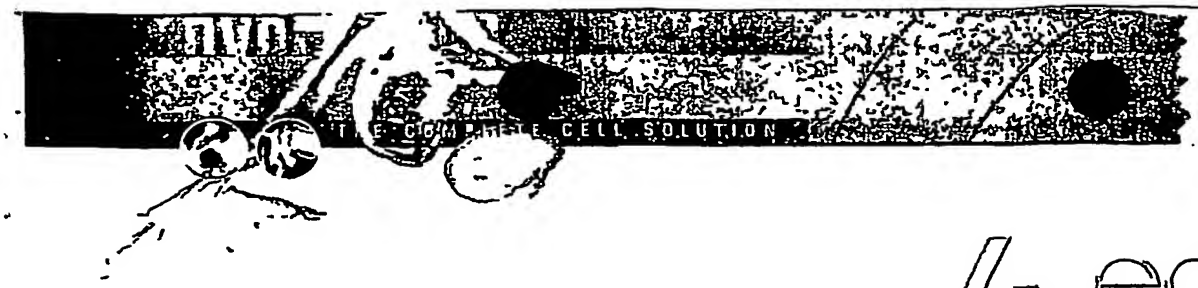
$$TCID_{50} = \frac{1}{10^{-3}} \times 10^{(1+01.25)}$$

$$= 10^{3.25}$$

Overall Result	Virus Present

*** End of Certificate***

Authorised by ..... **ECACC, Head of Quality** 4/12/00 **Date**



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of Cell Cultures

Centre for Applied Microbiology and Research
&
European Collection of Cell Cultures

This document certifies that Virus
(Deposit Ref V00120707) has been accepted as a patent deposit,
in accordance with
The Budapest Treaty of 1977,
with the European Collection of Cell Cultures on 7TH December 2000

Dr P J Packer
Quality Manager, ECACC

European Collection of Cell Cultures CAMR Salisbury Wiltshire SP4 0JG UK
Tel 44 (0) 1980 612512 Fax 44 (0) 1980 611315 Email ecacc@camr.org.uk Web Site ecacc.org.uk

BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

TO

BAVARIAN NORDIC RESEARCH
INSTITUTE GMBH
FRAUNHOFERSTRASSE 18B
D-82152 MARTINSRIED
GERMANY

NAME AND ADDRESS
OF DEPOSITOR

I IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY
MVA-575	V00120707

II SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

The microorganism identified under I above was accompanied by

[X] A scientific description

[ ] A proposed taxonomic designation

(Mark with a cross where applicable)

III RECEIPT AND ACCEPTANCE

This International Depository Authority accepts the microorganism identified under I above, which was received by it on 7TH December 2000 (date of the original deposit)[1]

IV RECEIPT OF REQUEST FOR CONVERSION

The microorganism identified under I above was received by this International Depository Authority on (date of the original deposit) and
A request to convert the original deposit to a deposit under the Budapest Treaty was received by it on (date of receipt of request for conversion)

IV INTERNATIONAL DEPOSITORY AUTHORITY

Name Dr P J Packer

Address ECACC
CAMR
Porton Down
Salisbury SP4 OJG

Signature(s) of person(s) having the power to represent the International Depository Authority or of authorized officials(s)

Date 23/5/01

1 Where Rule 6 4(d) applies, such date is the date on which the status of international depositary authority was acquired

BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

TO

BAVARIAN NORDIC RESEARCH
INSTITUTE GMBH
FRAUNHOFERSTRASSE 18B
D-82152 MARTINSRIED
GERMANY

VIABILITY STATEMENT
Issued pursant to Rule 10 2 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified on the following page

NAME AND ADDRESS OF THE PARTY
TO WHOM THE VIABILITY OF STATEMENT
IS ISSUED

I DEPOSITOR	II IDENTIFICATION OF THE MICROORGANISM
Name BAVARIAN NORDIC RESEARCH INSTITUTE GMBH Address FRAUNHOFERSTRASSE 18B D-82152 MARTINSRIED GERMANY	Accession number given by the INTERNATIONAL DEPOSITORY AUTHORITY 00120707 Date of the deposit or of the transfer[1] 7TH December 2000

II VIABILITY STATEMENT

The viability of the microorganism identified under II above was tested on[2]. On that date, the said microorganism was

☐[3] viable

☐[3] no longer viable

1 Indicate the date of the original deposit or, where a new deposit or a transfer has been made, the most relevant date (date of the new deposit or date of the transfer)

2 In the cases referred to in Rule 10 2 (a) (ii) and (iii), refer to the most recent viability test

3 Mark with a cross the applicable box

IV CONDITIONS UNDER WHICH THE VIABILITY TEST HAS BEEN PERFORMED [4]	
MVA-575 - V00120707 THIS VIRUS WAS TITRATED ON BHK CELLS $TCID_{50} = 10^{6.5}$	
V INTERNATIONAL DEPOSITARY AUTHORITY	
Name Dr P J Packer ECACC CAMR Address Porton Down Salisbury Wiltshire SP4 0JG	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s) Date 23/3/01 PSPacker

4 Fill in if the information has been requested and if the results of the test were negative

Certificate of Analysis

Product Description MVA-575
Accession Number 00120707

Test Description Determination of $TCID_{50}$ of cytopathic Virus titration (SOP ECACC/055) Cell

Acceptance Criterion/Specification/Criteria Negative controls should show no sign of Cytopathic effects The Test Sample is serially diluted into in 4 wells of indicator cell lines for each dilution Cytopathic effects indicate that virus is present Virus titre is calculated using the below equation where x is the value obtained from a standard $TCID_{50}$ Table as a result of the distribution of the wells displaying less than 4 positive wells per dilution, and y is the value of the highest dilution where all 4 wells are positive

$$TCID_{50} = \frac{1}{y} \times 10^{1+x}$$

Date	19/01/01	
Result	Indicator Cell Line	BHK 21 CLONE 13
	Negative Control	NO CPE
	Test Sample	CPE
	Distribution of less that 4 positive wells	4, 4, 0
	X	0 50
	Y	10^{5}

$$TCID_{50} = \frac{1}{10^{5}} \times 10^{1+0.50}$$

$$= 10^{6.5}$$

Overall Result Virus Present

Test Description The Detection of Mycoplasma by Isolation on Mycoplasma Pig Serum Agar and in Mycoplasma Horse Serum Broth
SOP QC/MYCO/01/02

Acceptance Criterion/Specification All positive controls (*M pneumoniae & M orale*) must show evidence of mycoplasma by typical colony formation on agar plates Broths are subcultured onto Mycoplasma Pig Serum Agar where evidence of mycoplasma by typical colony formation is evaluated All negative control agar plates must show no evidence of microbial growth
The criteria for a positive test result is evidence of mycoplasma by typical colony formation on agar A negative result will show no such evidence

Test Number	21702	
Date	12/02/01	
Result	Positive Control	Positive
	Negative Control	Negative
	Test Result	Negative
	Overall Result	PASS

Authorised by RSAL **ECACC, Head of Quality** 5/3/01 **Date**

Certificate of Analysis

Product Description MVA-575
Accession Number 00120707

Test Description Detection of Mycoplasma using a Vero indicator cell line and Hoechst 33258 fluorescent detection system
SOP QC/MYCO/07/05

Acceptance Criterion/Specification The Vero cells in the negative control are clearly seen as fluorescing nuclei with no cytoplasmic fluorescence Positive control (*M orale*) must show evidence of mycoplasma as fluorescing nuclei plus extra nuclear fluorescence of mycoplasma DNA Positive test results appear as extra nuclear fluorescence of mycoplasma DNA Negative results show no cytoplasmic fluorescence

Test Number 21702

Date 12/02/01

Result

Positive Control	Positive
Negative Control	Negative
Test Result	Negative
Overall Result	PASS

Test Description Detection of bacteria and fungi by isolation on Tryptone Soya Broth (TSB) and in Fluid Thioglycollate Medium (FTGM) SOP QC/BF/01/02

Acceptance Criterion/Specification All positive controls (*Bacillis subtilus Clostridium sporogenes* and *Candida albicans*) show evidence of microbial growth (turbidity) and the negative controls show no evidence of microbial growth (clear)
The criteria for a positive test is turbidity in any of the test broths All broths should be clear for negative test result

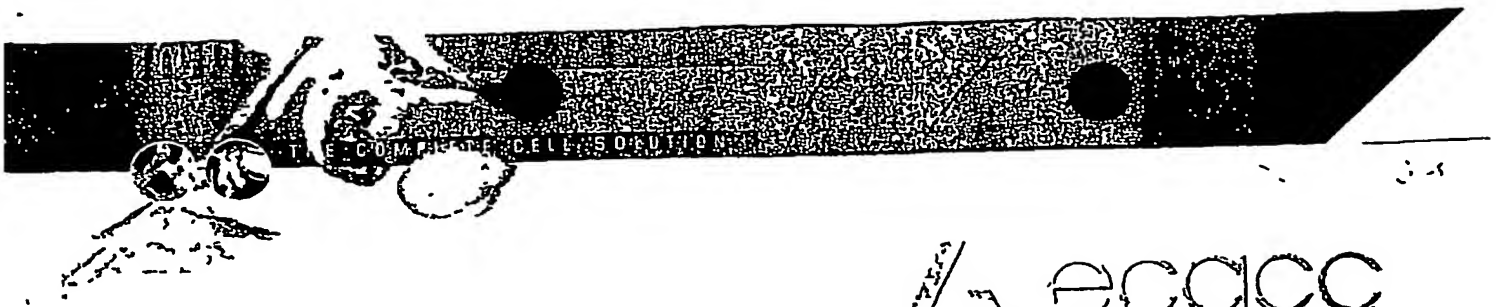
Test Number 21702

Date 12/02/01

Result

Positive Control	Positive
Negative Control	Negative
Test Result	Negative
Overall Result	PASS

Authorised by [signature] **ECACC, Head of Quality** 5/3/01 **Date**



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of Cell Cultures

Centre for Applied Microbiology and Research
&
European Collection of Cell Cultures

This document certifies that Virus
(Deposit Ref V99101431) has been accepted as a patent deposit,
in accordance with
The Budapest Treaty of 1977,
with the European Collection of Cell Cultures on 14TH October 1999

PJ Packer

Dr P J Packer
Quality Manager, ECACC

European Collection of Cell Cultures CAMR Salisbury Wiltshire SP4 0JG UK
Tel 44 (0) 1980 612512 Fax 44 (0) 1980 611315 Email ecacc@camr.org.uk Web Site ecacc.org.uk

BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

TO
PROF DR DR H C MULT ANTON MAYR
WEILHEIMER STR 1
D-82319 STARNBERG
GERMANY

NAME AND ADDRESS
OF DEPOSITOR

I IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR VERO-MVA	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY V99101431

II SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

The microorganism identified under I above was accompanied by

[X] A scientific description

[ ] A proposed taxonomic designation

(Mark with a cross where applicable)

III RECEIPT AND ACCEPTANCE

This International Depository Authority accepts the microorganism identified under I above, which was received by it on 14TH October 1999 (date of the original deposit)[1]

IV RECEIPT OF REQUEST FOR CONVERSION

The microorganism identified under I above was received by this International Depository Authority on (date of the original deposit) and
A request to convert the original deposit to a deposit under the Budapest Treaty was received by it on (date of receipt of request for conversion)

IV INTERNATIONAL DEPOSITORY AUTHORITY

Name Dr P J Packer

Address ECACC
CAMR
Porton Down
Salisbury SP4 0JG

Signature(s) of person(s) having the power to represent the International Depository Authority or of authorized officials(s)

Date 21.10.99

1 Where Rule 6.4(d) applies, such date is the date on which the status of international depositary authority was acquired

1991

Form BP/4 (sole page)

BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

TO

PROF DR DR H C MULT ANTON MAYR
WEILHEIMER STR 1
D-82319 STARNBERG
GERMANY

VIABILITY STATEMENT
Issued pursant to Rule 10 2 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified on the following page

NAME AND ADDRESS OF THE PARTY
TO WHOM THE VIABILITY OF STATEMENT
IS ISSUED

I DEPOSITOR	II IDENTIFICATION OF THE MICROORGANISM
Name PROF DR DR H C MULT ANTON MAYR Address WEILHEIMER STR 1 D-82319 STARNBERG GERMANY	Accession number given by the INTERNATIONAL DEPOSITORY AUTHORITY V99101431 Date of the deposit or of the transfer 14TH October 1999
II VIABILITY STATEMENT	
The viability of the microorganism identified under II above was tested on [2] On that date, the said microorganism was [X] [3] viable [ ] [3] no longer viable	

1 Indicate the date of the original deposit or, where a new deposit or a transfer has been made, the most relevant date (date of the new deposit or date of the transfer)

2 In the cases referred to in Rule 10 2 (a) (ii) and (iii), refer to the most recent viability test

3 Mark with a cross the applicable box

Form BP/4 (first page)

IV CONDITIONS UNDER WHICH THE VIABILITY TEST HAS BEEN PERFORMED [4]

VERO-MVA - 99101431

THE VIRUS WAS GROWN ON VERO CELLS ACCORDING TO THE DEPOSITORS INSTRUCTIONS THE VIRUS WAS VIABLE PRODUCING CYTOPATHIC EFFECT AFTER 48 HOURS A LITRE OF 6 X 10^6 PLAQUE FORMING UNITS/ML WAS OBTAINED

V INTERNATIONAL DEPOSITARY AUTHORITY

Name: Dr P J Packer ECACC CAMR Address: Porton Down Salisbury Wiltshire SP4 0JG	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s) Date 8/3/01 P.S Packer

4 Fill in if the information has been requested and if the results of the test were negative